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13. SUPPLEMENTARY NOTES

14. ABSTRACT: We proposed that decorating ovarian tumor cells with αGal (using RGD*-αGal) will lead to their destruction by patients' naturally occurring antibody against αGal. We demonstrated staining of αGal^+ tumor cells with FITC-conjugated IB4 lectin, as well as with human serum and secondary antibody. We also developed an in vitro assay to measure complement-dependent cytotoxicity (CDC) of tumors expressing αGal. We showed in mice that human serum containing anti-αGal antibody induced regression of αGal -expressing L5178Y tumor. Then we tested if αGal^- tumor cells that express $\alpha_V\beta_3$ integrin will bind RGD*-αGal (via RGD*) to the $\alpha_V\beta_3$ integrin to express sufficient αGal to allow their destruction by anti-Gal antibody and complement. Tumor cells expressing the $\alpha_V\beta_3$ integrin were treated with RGD*-αGal, followed by direct staining with FITC-IB4 lectin or with anti-αGal antibody and tested for indirect detection with anti-Gal antibody or for CDC. While detection of αGal was seen at low levels in some experiments, we were not able to reproducibly show sufficient levels to enable translation into clinical treatment simulation in vitro or in mice. These results suggest that we should be pursuing means to further increase the αGal expression we can induce on these tumors prior to trying to proceed to model treatment strategies in vitro or in vivo. Novel approaches towards this goal have been proposed and are detailed in the "linked" annual report being submitted by our co-investigator, Dr. Laura Kiessling.

15. SUBJECT TERMS
Alpha-Gal, complement-mediated killing, ovarian cancer, antitumor effect

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PROGRESS REPORT

a. Introduction

As outlined in the Statement of Work, during this first year (September 15, 2008 – September 14, 2009) we have been working on Aim 1 of the proposal "Characterize the mechanisms of the destruction of RGD*-αGal labeled OVCAR-3 and MOSEC tumor by anti-Gal in vitro" and Aim 2 "Establish murine models to enable preclinical development of RGD*-αGal".

b. Body.

In studies related to Aim 1, we first developed in vitro assays for detecting αGal on the surface of tumor cells. We tested several αGal^+ mouse (L5178Y lymphoma, MOSEC ovarian carcinoma) and αGal^- human tumor cell lines (M21 and WM115 melanomas, OVCAR-3 and 7, SKOV3, CAOV3 ovarian carcinomas). In addition we used mouse B16 melanoma cells transfected with α1,3galactosyltransferase to express αGal (B16-αGal). This cell line was generated by our collaborator, Dr. Uri Galili (1) and obtained from him along with the αGal^- parental B16 cell line. Using flow cytometry, we have demonstrated staining of αGal^+ tumor cells with FITC-conjugated IB4 lectin which selectively binds αGal (2). Figure 1 shows a representative experiment using B16-αGal and control B16 cells. Similar positive staining of αGal^+ tumor cells, in contrast to αGal^- cells, was achieved by incubating the cells with human serum (or anti-αGal monoclonal antibody) followed by biotin-conjugated anti-IgM and streptavidin-PE (data not shown). These experiments were repeated several times with the tumor cell lines listed above and consistent results were obtained.

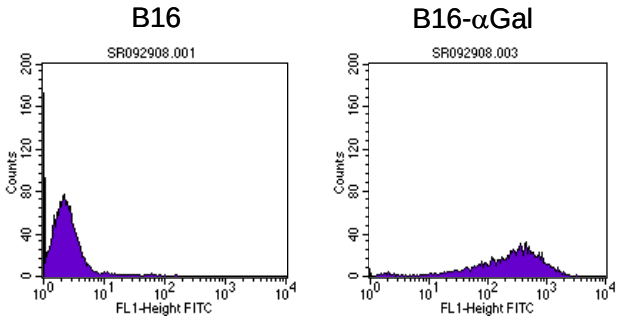

Figure 1. **Detection of αGal expression by staining with IB4 lectin**. Control B16 cells and B16 cells transfected with α1,3galactosyltransferase to express αGal (B16-αGal) were stained with αGal-specific FITC-IB4 lectin. Only B16-αGal showed positive staining.

In the Statement of Work we proposed that decorating ovarian tumor cells with αGal (using RGD*-αGal) will lead to their destruction by patients' naturally occurring antibody against αGal. Therefore, having established clinically-relevant assays for detecting αGal, we tested if treating αGal^- tumor cells that express $\alpha_V\beta_3$ integrin will bind RGD*-αGal (via RGD*) to the $\alpha_V\beta_3$ integrin expressed on tumor cells and therefore result in αGal expression on their cell surface. Treatment of M21 tumor cells expressing the $\alpha_V\beta_3$ integrin (3) with RGD*-αGal, followed by staining with either FITC-IB4 lectin (Figure 2) or anti-αGal antibody (data not shown) did not reproducibly show detectible levels of αGal expression on the cell surface. Similar results were obtained using ovarian cell lines treated with RGD*-αGal. Prior studies have indicated that some RGD*-αGal could bind at low levels to certain tumors that express particularly high levels of $\alpha_V\beta_3$ integrin (3). These recent results suggest to us that we should be pursuing means to further increase the αGal expression we can induce on these tumors prior to trying to proceed to modeling treatment strategies in vitro or in vivo. Approaches towards this goal are mentioned below and are further detailed in the "linked" annual report being submitted by our co-investigator, Dr. Laura Kiessling.

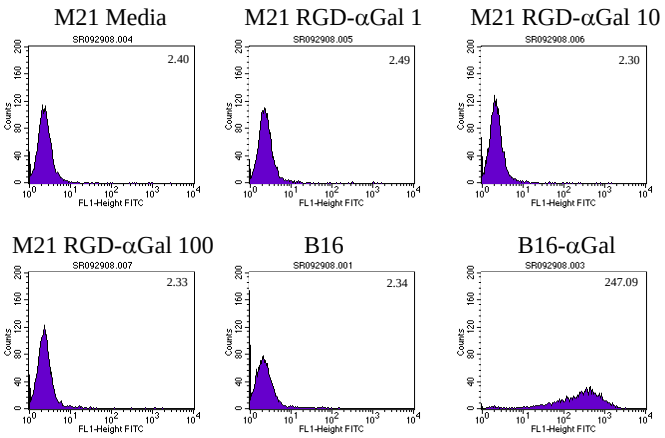

Figure 2. **Treatment of M21 cells with RGD-αGal does not result in detectable αGal expression**. M21 cells were incubated with the RGD-αGal (1, 10 or 100 nM) in the activation buffer followed by staining with αGal-specific FITC-IB4 lectin. B16 cells and B16-αGal cells were stained with FITC-IB4 lectin as a negative and positive control, respectively. The results show no staining of M21 cells, treated with RGD-αGal, with FITC-IB4 lectin.

In studies related to Aim 1, we also developed in vitro assays to measure complement-dependent cytotoxicity (CDC) of tumors expressing αGal on their surface. First, we compared a flow cytometric assay using propidium iodide (PI) for staining dead cells and conventional 51-chromium assay for measuring CDC of 14.18-IL2 fusion protein against M21 cells (positive control for antibody-dependent cytotoxicity used in our laboratory) and found both these methods to be equally effective. Then, using the PI test, we repeatedly demonstrated that αGal^+ L5178Y cells, MOSEC cells and B16-αGal^+ cells, but not αGal^- B16 cells, were effectively killed after incubating with human serum (HS) as a source of both anti-αGal antibody and complement.

While treatment of M21 tumor cells with RGD*-αGal followed by incubating with human serum as a source of both anti-αGal antibody and complement did not reproducibly show cell killing above background levels in this clinically relevant assay, substantial killing was obtained using 14.18-IL2 as a positive control (Figure 3). Similar results were obtained with ovarian cell lines.

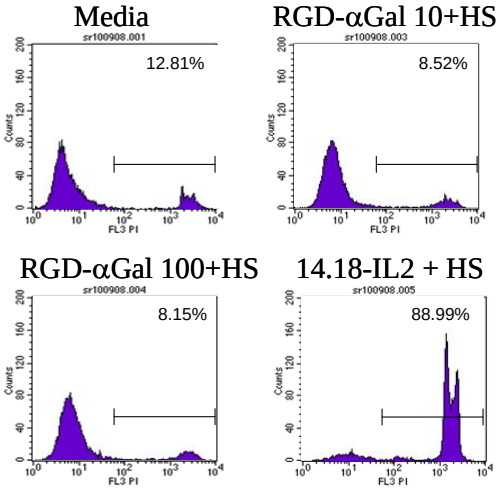

Figure 3. **CDC of M21 tumor cells**. M21 cells were incubated with the RGD-αGal* (1, 10 or 100 nM; 10 and 100 nM are shown) in the activation buffer followed by incubating with HS (40 min at 4°C and 60 min at 37°C). As a positive control, M21 cells were incubated with 14.18-IL2 (40 min at 4°C) and HS (60 min at 37°C). 14.18 is a mAb that recognizes GD2 on the surface of M21 cells. M21 cells were then treated with PI to stain dead cells. The numbers in the histograms indicate percentage of PI^+ cells. The results in this experiment show no CDC of M21 cells treated with RGD-αGal, but do show killing with 14.18 mAb. Testing of this concept using Antibody Dependent Cell-mediated Cytotoxicity (ADCC) is still pending, and ADCC may be more sensitive in induction of killing in the presence of a small amount of bound anti-Gal antibody. Even so, these studies provide further support for our plans to increase the amount of αGal that we can be getting onto the surface of tumor cells, especially ovarian cancer cells. Strategies we are pursuing with Dr. Kiessling are detailed in her progress report and outlined below.

In studies related to Aim 2 we evaluated in vivo whether anti-αGal antibody in human serum could induce regression of αGal-expressing tumor. L5178Y lymphoma cells (αGal^+) were implanted subcutaneously in syngeneic DBA/2 mice and injected intra-tumorally (i.t.) with human serum or PBS (control) 7 days later. The results in Figure 4 show that treatment with human serum induced complete tumor regression in 83.3% of mice compared with 16.6% in control group. As the major antibody target that should be recognized by the human serum on the L5178Y tumor cells is the αGal^+ , these data support the hypothesis that this in vivo system is a

reasonable means for detecting in vivo anti-tumor effects mediated by anti-Gal antibody. Currently we are confirming these data in experiments using B16-αGal tumors vs. parental B16 tumors in C57BL/6 mice.

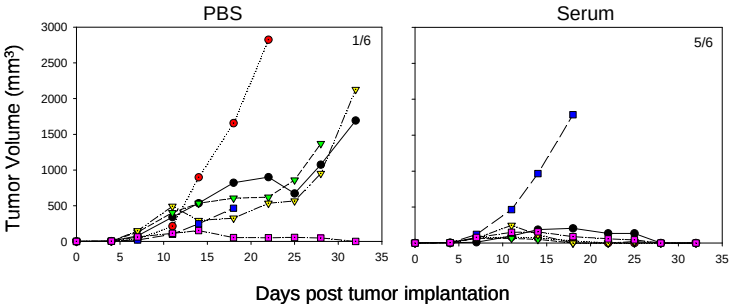

Figure 4. **Effect of human serum on L5178Y tumor growth**. DBA/2 mice were implanted s.c. with L5178Y tumor cells and treated i.t. on day 7 with PBS or human serum. Tumor volumes of individual mice are shown. The numbers in the upper right hand corner indicate tumor-free/total mice.

c. Key research accomplishments

- Two in vitro systems for detecting αGal on cell surface have been developed
- Two in vitro assays for complement-mediated cell cytotoxicity of αGal-expressing cells have been developed
- αGal^+ tumor cells, including MOSEC, but not αGal^- tumor cells, including OVCAR-3, are killed by human serum in vitro, indicating effective lysis by anti-Gal antibody.
- In vitro treatment of αGal^- tumor cells with RGD*-αGal compound did not result in reproducible detection of αGal on cell surface or in complement-mediated cell killing by human serum, supporting our plans to enhance passive expression of αGal on human tumor cells via additional novel strategies.
- In vivo treatment of αGal-expressing tumors with human serum induced complete tumor regression
- Purification of anti-Gal antibody from human serum, done collaboratively with Dr. Galili.

d. Reportable outcomes

- No manuscripts were published during this first year.

e. Conclusions

- αGal on the surface of tumor cells can be reproducibly detected using two separate methods.
- Human serum kills αGal^+ but not αGal^- tumor cells via complement-mediated killing.
- Treatment with human serum induces regression of αGal^+ L5178Y cells in vivo.
- Treatment of αGal^- tumor cells with RGD*-αGal did not result in reproducible αGal expression or complement-mediated cell killing by human serum in our clinically relevant in vitro assays.
- The results suggest that RGD*-αGal, as currently formulated, and used as a single agent, does not induce sufficient αGal expression to enable tumor killing by anti- αGal antibody and complement under conditions that might simulate the in vivo (clinical) setting.
- Testing of this concept using Antibody Dependent Cell-mediated Cytotoxicity (ADCC) is still pending, and may be more sensitive.
- Two novel compounds are proposed to increase αGal expression on tumor cells:
    i. RGD*-αGal "dendrimers". These are synthetic molecules that link several αGal molecules to each RGD*, thereby multiplying 3-5 fold the amount of αGal that can be placed onto the surface of $\alpha_V\beta_3$ integrin bearing tumor cells. These RGD*-αGal dendrimers should bind to the integrins on the ovarian cancer cell and deposit far more αGal on the cell surface, to facilitate greater binding by anti-Gal antibody (and greater complement mediated destruction).

ii. CD13L-αGal bifunctional ligands. In order to enhance the binding and destruction by anti-Gal antibody, we are working with Dr Kiessling to augment the deposition of αGal onto ovarian cancer cells, by linking αGal to a separate ligand (CNGRCG) with selective binding to the CD13 (aminopeptidase N) that is overexpressed on many tumor cell surfaces. This new CD13L-αGal bifunctional ligand is now being synthesized in Dr. Kiessling's laboratory. We will be testing it in our in vitro systems (alone, in combination with RGD*-αGal bifunctional ligands and with RGD*-αGal dendrimers, for the induction of detectible αGal on the surface of tumor cells and for induction of lysis by the anti-Gal antibody and Complement in HS.

iii. Dr. Kiessling's lab is working on the synthesis of these and we are preparing our in vitro and in vivo assays to be testing the development of these in year 2.

- Finally, in a related project that is conceptually linked to this preclinical project with Drs. Kiessling and Galili, we are working with Dr. Galili and our UW colleague Dr. Mark Albertini to open a separate clinical trial of therapy here at the UWCCC. This clinical trial (A Phase I Study to Evaluate the Toxicity and Feasibility of Intratumoral Injection of αGal Gylycolipids in Patients with Advanced Melanoma) is approved by the FDA and already open at the University of Massachusetts; it is currently under review by our UWCCC review committee in preparation for IRB submission, and should be open to accrual here at UW this winter. It is providing the clinical pathway for clinical treatment and monitoring of anti-Gal directed immunotherapy, that would be used for further development of the anti-Gal preclinical work being pursued in this Department of Defense project with Dr. Kiessling.

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